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The PTH/PTHrP Receptor Can Delay Chondrocyte Hypertrophy In Vivo without Activating Phospholipase C

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Summary

One G protein-coupled receptor (GPCR) can activate more than one G protein, but the physiologic importance of such activation has not been demonstrated in vivo. We have generated mice expressing exclusively a mutant form of the PTH/PTHrP receptor (DSEL) that activates adenylyl cyclase normally but not phospholipase C (PLC). DSEL mutant mice exhibit abnormalities in embryonic endochondral bone development, including delayed ossification and increased chondrocyte proliferation. Analysis of the differentiation of embryonic metatarsals in vitro shows that PTH(1–34) and forskolin inhibit, whereas active phorbol ester stimulates, hypertrophic differentiation. Thus, PLC signaling via the PTH/PTHrP receptor normally slows the proliferation and hastens the differentiation of chondrocytes, actions that oppose the dominant effects of PTH/PTHrP receptors and that involve cAMP-dependent signaling pathways.

Introduction

The parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor is a member of the B subfamily of G protein-coupled receptors (GPCRs; Gether, 2000; Wess, 1998). This receptor can activate at least three types of G proteins: G_s, which activates adenylyl cyclase (AC)/protein kinase A (PKA), the G_q/G_{11} family, which activates phospholipase C (PLC)/protein kinase C (PKC), and G_i, which can inhibit adenylyl cyclase and exert other actions as well (Bringhurst et al., 1993; Fujimori et al., 1992; Offermanns et al., 1996; Rappaport and Stern, 1986; Schneider et al., 1994). Utilizing these pathways, the PTH/PTHrP receptor can concurrently generate multiple intracellular second messengers, including cAMP, inositol triphosphate (IP_3), diacylglycerol, and cytosolic Ca^{2+} transients, which then may mediate specific cellular responses in target cells (Friedman et al., 1996; Guo et al., 2001; Miao et al., 2001; Radeff et al., 2001; Swarthout et al., 2001; Zhen et al., 2001). Although such multiple signaling commonly is observed with other B family receptors and with receptors in the A and C families of GPCRs as well (Francesconi and Duvoisin, 1998; Gether, 2000; Gudermann et al., 1996; Kukkonen et al., 2001; Pisegna and Wank, 1996; Wess, 1998), the functional significance of this diverse signaling has not been established in vivo. This is crucial, however, since the occurrence and intensity of specific signaling responses to GPCRs are not uniquely determined by the receptor sequence but depend also upon many other factors that may be controlled directly or indirectly by the cell's environment in vivo. These may include the level of receptor expression, the abundance of specific G proteins and other adaptor or scaffolding proteins, the expression and activity of downstream kinases and their substrates, functional interactions with signals generated by ligands activating other types of receptors expressed in the same target cell, interactions with adjacent cells, constituents of the extracellular matrix, mechanical forces in the tissue, and local or systemic regulatory factors. For these reasons, interdiction of specific signaling events within the context of an otherwise normal intact animal is a critical step toward understanding the physiologic roles of such multiple receptor signals.

Chondrocytes and osteoblasts are the primary target cells for PTHrP and PTH in bone, and both cell types express PTH/PTHrP receptors that regulate their differentiation and function (Amizuka et al., 1999; Bos et al., 1996; Langub et al., 2001; Lee et al., 1995). Chondrocytes in the murine fetal growth plate, in response to PTHrP expressed in the periarticular region (and perhaps to circulating PTH as well), show continued proliferation and delayed hypertrophy, effects that are disrupted by ablation of the PTH/PTHrP receptor (Karaplis et al., 1994; Lanske et al., 1996). Periarticular expression of PTHrP in fetal growth plates is stimulated by Indian hedgehog (Ihh) synthesized by postmitotic, prehypertrophic chondrocytes that also express abundant PTH/PTHrP receptors (Lee et al., 1995). By prolonging the proliferation of chondrocytes, PTHrP delays differentiation into cells that synthesize Ihh, thereby establishing a negative feedback loop that controls the pace of chondrocyte differentiation in the growth plate (Chung et al., 1998; St-Jacques et al., 1999; Vortkamp et al., 1996). In skeletons of adult animals, PTH increases the lineage commitment, differentiation, and function of osteoblasts, especially those adjacent to trabecular bone (Dobnig and Turner, 1995; Hock and Gera, 1992; Miao et al., 2001; Mitlak et al., 1996; Nishida et al., 1994), and it also increases the formation and activity of bone-resorbing osteoclasts (Dobnig and Turner, 1997; Hock and Gera, 1992; Kitazawa et al., 1991; Tam et al., 1982; Uzawa et al., 1995).

Previous efforts to identify the particular intracellular signaling pathways whereby PTH/PTHrP receptors regulate the differentiation, proliferation, and function of chondrocytes and osteoblasts have been conducted mainly with in vitro cell or organ culture systems, in which pharmacologic agonists or antagonists of G_s-, $G_{q/11}$-, or G_i-dependent responses could be introduced directly. Various analogs of PTH believed to signal selectively via PTH/PTHrP receptors, such as PTH(1–31), PTH(3–34), PTH(7–34), or PTH(28–48) (Fujimori et al., 1991; Jouishomme et al., 1994; Takasu et al., 1999a), also have been used, both in vitro and in vivo. Studies with isolated chondrocytes in vitro, for example, have

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suggested that PTH/PTHrP receptors may regulate expression of type X collagen, Bcl-2, and vitamin D receptors via activation of PKC (Klaus et al., 1994; Zhen et al., 2001), whereas the cAMP/PKA pathway may play a primary role in controlling mineralization and synthesis of alkaline phosphatase, glycosaminoglycans, Ihh, and retinol binding protein (Kato et al., 1988; Kawashima-Ohya et al., 1999; Shukunami et al., 1997; Yoshida et al., 2001). The relative roles of PKA versus PKC in regulating alkaline phosphatase may differ between populations of chondrocytes isolated from distinct regions of the growth plate, however (Schwartz et al., 1997), and investigators addressing the involvement of these kinases in mediating the proliferative response of chondrocytes to PTH have reached different conclusions (Klaus et al., 1994; Koike et al., 1990; Schluter et al., 1989; Zuscik et al., 1994).

We previously described a mutant form of the PTH/PTHrP receptor, in which a clustered DSEL sequence is substituted for the wild-type EKKY within the receptor's second intracellular loop. This mutation specifically interrupts signaling via the PLC/PKC pathway but preserves normal activation of adenylyl cyclase/PKA (Guo et al., 2001; Iida-Klein et al., 1997). Thus, porcine renal epithelial (LLC-PK1) cells stably transfected with DSEL mutant PTH/PTHrP receptors exhibited normal cAMP responsiveness to PTH but no PLC activation and no regulation of sodium phosphate cotransport, a response known to be PKC dependent in these cells (Iida-Klein et al., 1997). When expressed in clonal chondrocytes, in which endogenous PTH/PTHrP receptor expression previously had been ablated, these mutant (DSEL) receptors mediated normal (PKA-dependent) regulation of chondrocyte differentiation by PTH (Guo et al., 2001). To ascertain the importance of PLC-dependent PTH/PTHrP receptor signaling under physiologic conditions in vivo, in the absence of administered exogenous PTH, we have generated mice that express only the DSEL, PLC-defective form of the PTH/PTHrP receptor. Cells isolated from these animals fail to activate PLC via the PTH/PTHrP receptor and show abnormal development of endochondral bone during fetal life, including an early delay in hypertrophic differentiation, blood vessel invasion, and bone deposition, and a later expansion of columnar proliferating chondrocytes. This phenotype is quite distinct from that observed in mice lacking the PTH/PTHrP receptor altogether, and indicates that PLC signaling by this receptor normally serves to dampen the dominant chondrocyte response to PTHrP. Thus, these studies establish roles for activation of multiple G proteins by one G protein-coupled receptor in vivo.

Results

Generation and Analysis of DSEL Mice

A clustered mutation (DSEL) in the second intracellular loop of the PTH/PTHrP receptor, known to abrogate PLC but not adenylyl cyclase signaling (Iida-Klein et al., 1997), was inserted in place of the wild-type sequence EKKY on one allele of the receptor gene in J1 embryonic stem cells. In addition to the DSEL mutation, the targeting strategy employed produced a single residual loxP site, located within the intron between exons E1 and E2 of the receptor (Figure 1A). Each of four independent loxP/Cre-targeted clones produced chimeric animals and successfully contributed to germline transmission in C57BL/6 mice. Homozygotes were successfully derived from two independent DSEL founders, and similar phenotypes were observed in 129/SvJ/C57BL/6 genetic backgrounds. Heterozygous and homozygous DSEL mice were born in the expected Mendelian ratio (of 516 mice, 126 [24.4%] were homozygous mutants [$PTHR^{D/D}$], 122 [23.6%] were wild-type [$PTHR^{+/+}$], and 268 [51.9%] were heterozygotes [$PTHR^{+/D}$]). Unlike mice lacking PTH/PTHrP receptors, which die at birth or immediately thereafter (Lanske et al., 1996), homozygous $PTHR^{D/D}$ mutants are viable, appear grossly normal, and are fertile. Blood ionized calcium and serum phosphate of mutant mice, measured at birth or at 4 and 8 weeks thereafter, were normal (data not shown).

DSEL Mice Fail to Activate Phospholipase C via the PTH/PTHrP Receptor

Previous studies in vitro have shown that the DSEL mutation selectively abolishes PTH/PTHrP receptor activation of PLC, leaving adenylyl cyclase activation intact (Iida-Klein et al., 1997). To confirm defective PLC activation via mutant PTH/PTHrP receptors in $PTHR^{D/D}$ mice, we analyzed IP_3 generation following PTH treatment in vitro of primary proximal tubule cells obtained from $PTHR^{+/+}$, $PTHR^{+/D}$, and $PTHR^{D/D}$ mice. Proximal tubule cells were used for these assays because their robust PLC response provides a sensitive assay for the effect of the DSEL mutation. As expected, PTH-stimulated IP_3 generation was readily measurable in proximal tubule cells from wild-type and $PTHR^{+/D}$ mice but was undetectable in cells from homozygous $PTHR^{D/D}$ mice (Figure 1B). Cyclic AMP responses to PTH in all three genotypes were equivalent, and cell surface expression of mutant DSEL receptors was normal (Figure 1B).

Phenotype of Early Bone Development in DSEL Mice

To determine whether PLC signaling via the PTH/PTHrP receptor plays any role in normal skeletal development, we first performed combined Alcian blue/Alizarin red staining of the entire skeleton of E18.5 mice. A defect in ossification of the tail, digital, and metatarsal bones was observed in $PTHR^{D/D}$ embryos, seen as an absence or decrease in Alizarin red staining (Figure 1C). To better appreciate the abnormalities in development of these most distal bones and other limb bones, we examined them histologically. During chondrogenesis, chondrocyte hypertrophy normally is followed by blood vessel invasion and deposition of bone by osteoblasts. A delay in this process can be observed with either targeted overexpression of PTHrP or targeted expression of constitutively active PTH/PTHrP receptors in chondrocytes (Schipani et al., 1997; Weir et al., 1996). In contrast, chondrocyte differentiation is accelerated following ablation of either PTHrP or the PTH/PTHrP receptor (Karaplis et al., 1994; Lanske et al., 1996). In tibias and metatarsals from E14.5–E18.5 $PTHR^{D/D}$ embryos, expansion of proliferating chondrocytes and delays in both chondrocyte differentiation and subsequent vascular invasion and bone deposition were observed (Figure 2). All

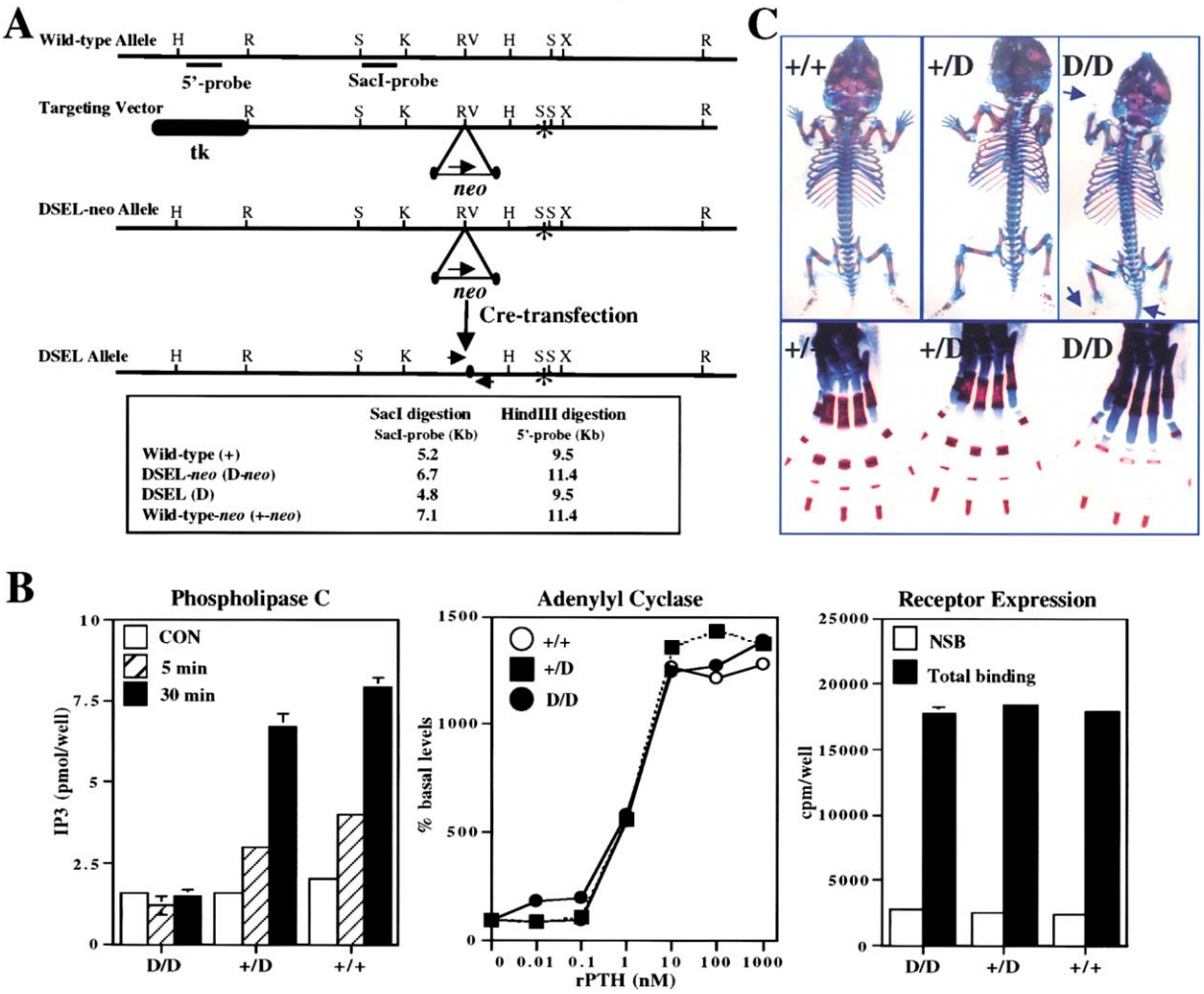

Figure 1. Generation and Characterization of DSEL Mutant Receptor in Mice

(A) Scheme of the genomic structure of the PTH/PTHrP receptor, the targeting vector, and DSEL-*neo* and DSEL alleles. In the targeting vector, the wild-type sequence EKKY in the second intracellular loop was mutated to DSEL (*), and a new recognition site for SacI also was introduced within the nucleotide sequence encoding the DSEL mutation. The loxP-flanked neomycin resistance cassette (*neo*) was inserted at an EcoRV site in the intron between exons E1 and E2 of the receptor gene. The DSEL-*neo* allele represents the structure of the PTH/PTHrP receptor locus following homologous recombination, whereas the DSEL allele illustrates the result of subsequent recombination of the two loxP sites (filled ovals) by Cre recombinase. Positions of PCR primers (5′-GATGGTACACCTACTGAGTGGTCG-3′/5′-AAGCGCTGCTAACCTCACACA GGT-3′) used for the detection of both wild-type and DSEL alleles are indicated by arrows, and expected sizes of PCR products were 145 and 181 bp for the wild-type and DSEL alleles, respectively, which differ only by one loxP sequence. The location of the probes used for Southern analysis also are shown (5′ probe and SacI probe); H, HindIII; S, SacI; X, XhoI; K, KpnI; R, EcoRI; RV, EcoRV; tk, PGK-thymidine kinase cassette. The table lists the predicted sizes of restriction fragments following SacI or HindIII digestion of each allele.

(B) Analysis of receptor signaling in wild-type and DSEL mice. Primary cultures of proximal tubule cells from wild-type (+/+), $PTHR^{+/D}$ heterozygote (+/*D*), and $PTHR^{D/D}$ homozygote (*D*/*D*) mice were used to measure PLC (left panel) and cAMP (middle panel) responses to rPTH(1–34), as well as surface expression of PTH/PTHrP receptors (right panel). For measurement of PLC stimulation, cells were exposed to rPTH(1–34) (100 nM) for 0 min (open bars), 5 min (hatched bars), or 30 min (filled bars) in the presence of 10 mM LiCl. Cell-associated cyclic AMP was measured after incubation of cells (wild-type, open circles; $PTHR^{+/D}$, filled squares; $PTHR^{D/D}$, filled circles) for 20 min in the presence of 3-isobutyl-1-methylxanthine (0.5 mM) and rPTH(1–34) at the indicated concentrations. Receptor expression was measured in the presence (filled bars) or absence (open bars) of added first antibody (G48). Results are expressed as means ± SEM of triplicates of pmol/well of inositol trisphosphate (left panel), percent of basal cAMP response (middle panel), or cpm/well of cell-associated ^{125}I-labeled third antibody (right panel). Similar results were obtained in two experiments of each type.

(C) Skeletal phenotypes of E18.5 embryos. Embryos harvested at E18.5 were digested free of soft tissue and then stained for both Alizarin red (for mineralized structures) and Alcian blue (for cartilage). Upper panel: entire skeleton of a wild-type, $PTHR^{+/D}$ heterozygote, and $PTHR^{D/D}$ homozygote, showing a defect in mineralization of the tail and paws in $PTHR^{D/D}$ (arrows). Lower panel: paw of hindlimb skeleton.

examined bones formed by endochondral bone formation, including calcaneous, talus, ulna, lumbar vertebrae, and sternum (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/3/2/183/DC1), exhibited delays in hypertrophy and ossification similar to those found in the metatarsals and tibia. These changes are most evident early in the development of each bone and thus occur asynchronously in correspondence to the differing pace of development of these bones. These findings thus resemble those in mice with

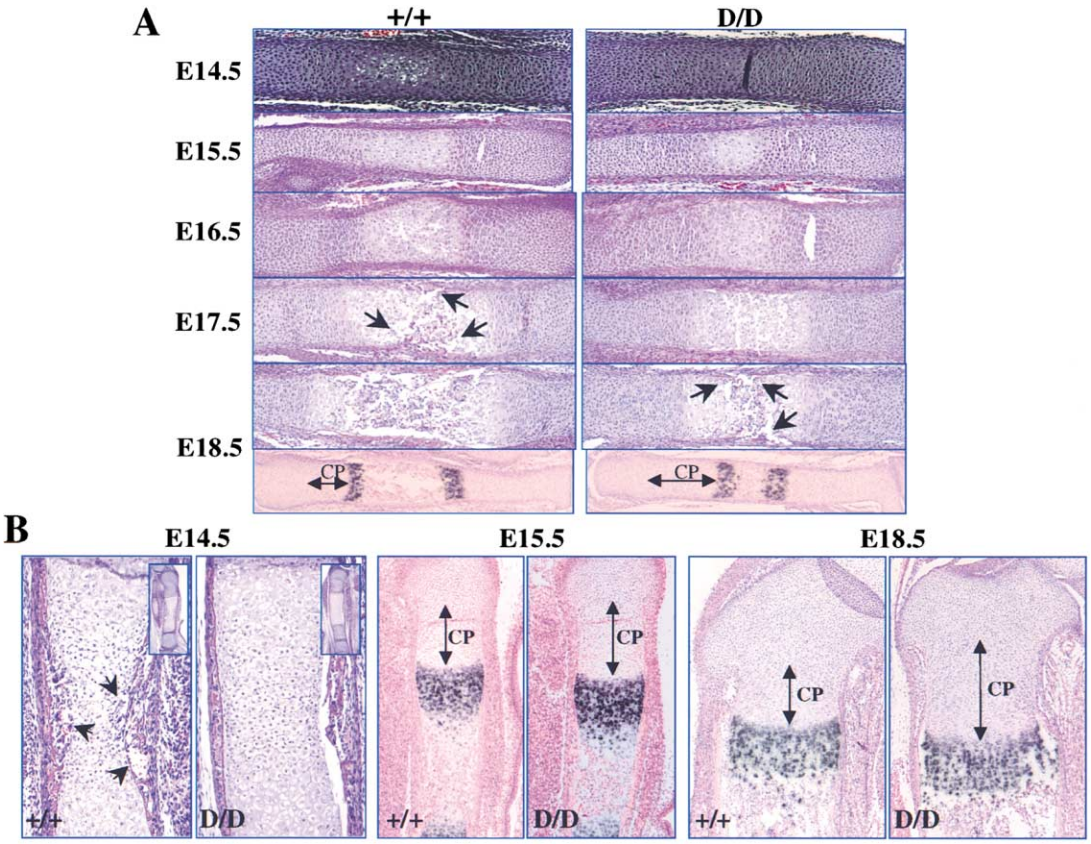

Figure 2. Histology of Tibial and Metatarsal Growth Plates

(A) Hematoxylin/eosin-stained longitudinal sections through wild-type (left panels) and *PTHR*$^{D/D}$ (right panels) metatarsals are shown for bones removed at days E14.5, E15.5, E17.5, and E18.5 (includes collagen X in situ hybridization) Sections are shown at 200× magnification (except E18.8, shown at 100×). At E17.5, blood vessel invasion (arrows) and degradation of hypertrophic chondrocytes was evident in wild-type but not yet in *PTHR*$^{D/D}$ metatarsals. CP, columnar proliferating chondrocytes.

(B) Hematoxylin/eosin-stained longitudinal sections through wild-type (+/+) and *PTHR*$^{D/D}$ mutant (*D/D*) tibiae at E14.5 (left panels, 200×), and in situ hybridization for collagen type X in tibia at E15.5 (middle panels, 100×) and E18.5 (right panels, 100×). In each panel, proximal is at the top, distal at the bottom. At E14.5, vascular invasion and cartilage degradation are observed in wild-type (arrowheads) but not yet in *PTHR*$^{D/D}$ mutants (insets show the entire bone rudiments).

increased PTH/PTHrP receptor signaling in chondrocytes and suggest that activation of PLC signaling by the PTH/PTHrP receptor normally may oppose the dominant net response to receptor activation.

Increased Proliferation and Delayed Maturation Cause an Accumulation of Columnar Proliferating Chondrocytes in DSEL Mice

The expansion of columnar proliferating chondrocytes in the growth plates of DSEL mice might reflect an increased rate of proliferation of these chondrocytes. To test this hypothesis, we performed pulsed BrdU labeling in pregnant mice. Two hours after administration of BrdU in vivo at E16.5 or E18.5, the labeling index of cells in the proliferating chondrocyte layer of *PTHR*$^{D/D}$ mutant embryos was increased by more than 50% compared to that of wild-type littermates (Figures 3A–3C and data not shown). This finding indicates that increased proliferation contributes to the expansion of the zone of columnar proliferating chondrocytes.

The switch from a proliferative to a postproliferative state is a key determinant of the number of chondrocytes in the proliferative versus hypertrophic pools. The PTH/PTHrP receptor regulates this transition, as documented previously by the shortened columns of proliferating chondrocytes and acceleration of hypertrophy observed in growth plates of mice lacking either PTHrP or the PTH/PTHrP receptor (Karaplis et al., 1994; Lanske et al., 1996). To assess the rate of cellular transition from the proliferating to the hypertrophic stage, we injected pregnant mice with a single dose of BrdU 48 hr before sacrificing the animals to measure the appearance of previously labeled cells in the zone of hypertrophic chondrocytes. As expected, hypertrophic chondrocytes, which are postproliferative cells, did not directly incorporate BrdU, as shown for animals sacrificed 2 hr after in vivo BrdU administration (Figures 3D–3F). In wild-type growth plates analyzed 48 hr after in vivo BrdU administration at E14.5, almost 30% of the hypertrophic chondrocytes were labeled (Figure 3H). This labeling index was decreased by approximately 40% in the hypertrophic zone of homozygous *PTHR*$^{D/D}$ embryos (Figures 3G–3I). Similar results were found when BrdU was administered at E16.5. Given the previous observation

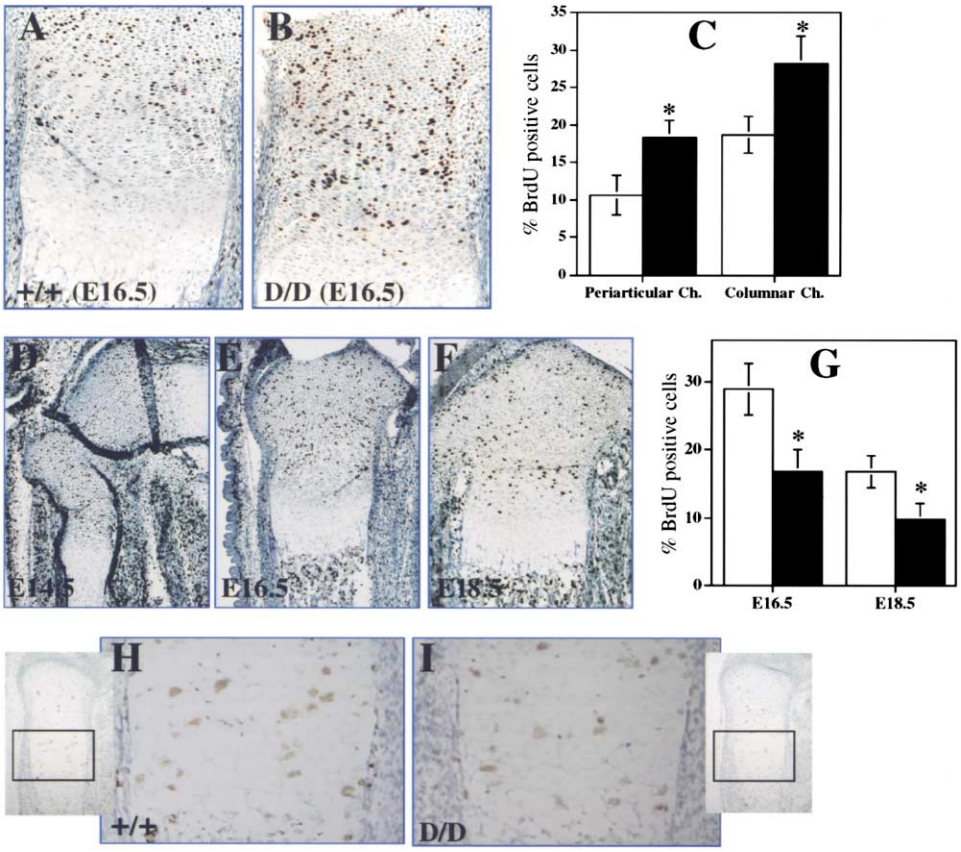

Figure 3. BrdU Labeling in Tibia Growth Plate Chondrocytes

BrdU was administered to pregnant mice either 2 hr (A, B, and D–F) or 48 hr (H and I) prior to sacrifice at the gestational ages shown. Immunohistochemical staining subsequently was performed on sections of the proximal tibiae of wild-type (A, D–F, and H) and *PTHR*$^{D/D}$ (B and I) embryos at E14.5 (D), E16.5 (A, B, E, H, and I), or E18.5 (F). BrdU-positive nuclei are stained dark brown. All nuclei are counterstained with methylene green. BrdU incorporation, calculated as percentage of BrdU-positive nuclei in the periarticular and columnar proliferating chondrocytes or in the hypertrophic chondrocyte layer, is shown graphically in (C) or (G), respectively. Bars represent means ± SD for wild-type (open bars) and *PTHR*$^{D/D}$ mutant (solid bars). *P $<$ 0.01 (Student's t test) versus wild-type.

of increased labeling of columnar proliferating chondrocytes, this result points to a considerable delay in hypertrophic differentiation of the proliferating chondrocytes as an additional contributor to the overall accumulation of columnar proliferating chondrocytes in DSEL mice. The delayed hypertrophic differentiation in DSEL mice is reflected by the reduced number of hypertrophic cells observed early in development (i.e., E14.5 and E15.5; Figure 2A). On the other hand, no significant change in the layer of hypertrophic chondrocytes was observed in more mature bones of DSEL mice, such as E18.5 metatarsals (Figure 2A) or E15.5 or E18.5 tibiae (Figure 2B), suggesting the possibility of an additional delay, in more mature bones, in removal of hypertrophic chondrocytes by vascular invasion and bone deposition by osteoblasts.

PLC-Independent Signaling Predominately Affects Chondrocyte Differentiation

To further analyze how specific signaling generated by the PTH/PTHrP receptor may affect chondrocyte differentiation, *PTHR*$^{+/-}$ and *PTHR*$^{D/-}$ mice were mated, and E15.5 metatarsal rudiments from the four expected genotypes in the resulting litters were cultured in serum-free medium in vitro. As shown in Figure 4A, mineralization of cartilage, as viewed serially with a dissecting microscope, gradually expanded distally from the center of the metatarsal rudiments of *PTHR*$^{+/-}$ embryos during 4 days in culture. In previous studies in vivo, hemizygous *PTHR*$^{+/-}$ mice exhibited normal growth plates (Lanske et al., 1996). In rudiments from *PTHR*$^{-/-}$ mice, mineralization was more extensive and extended much farther toward the articular surfaces, whereas the appearance of mineralized cartilage was greatly delayed in hemizygous *PTHR*$^{D/-}$ rudiments during this period of culture. The eventual appearance of mineralized cartilage after 4 days demonstrated that chondrocytes in these *PTHR*$^{D/-}$ rudiments ultimately were capable of hypertrophic differentiation. The pace of differentiation of heterozygous *PTHR*$^{+/D}$ rudiments was identical to those from *PTHR*$^{+/-}$ animals. As shown by in situ hybridization (Figure 4B), hypertrophic cells expressing collagen type X and osteopontin occupied large potions of the center of *PTHR*$^{+/-}$ rudiments, and this expression gradually extended distally. In the hemizygous *PTHR*$^{D/-}$ rudiments

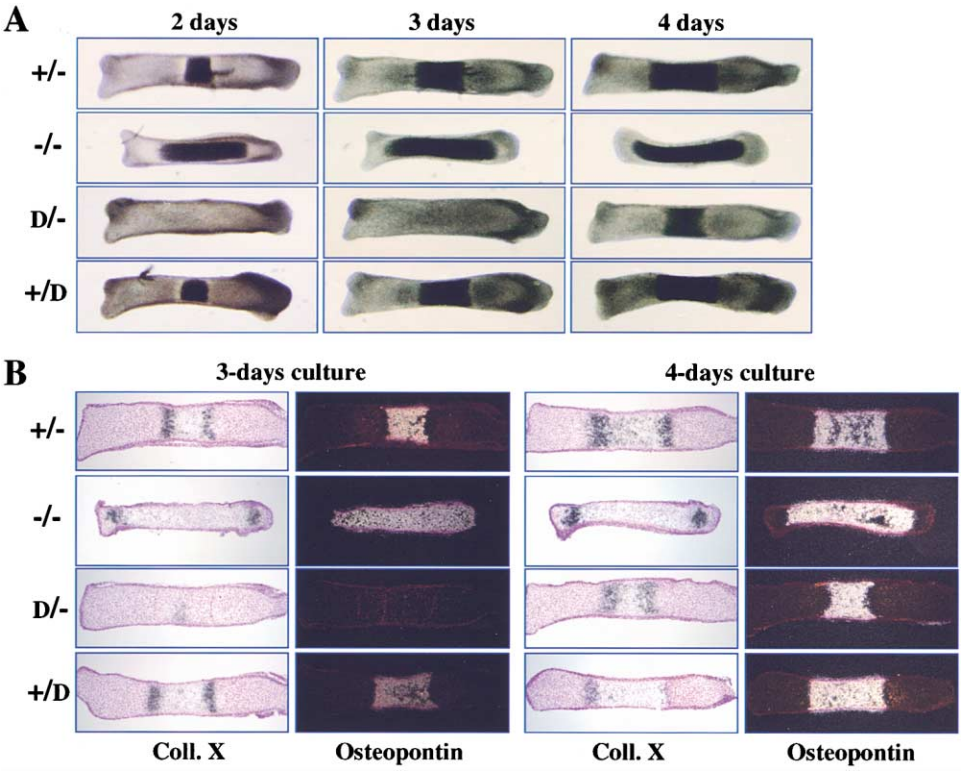

Figure 4. Development of Cultured Metatarsal Rudiments

Metatarsal bones from animals of the indicated genotypes were isolated and cultured for up to 4 days.

(A) Inverted microscopy (49× magnification) of E15.5 metatarsals cultured for 2, 3, or 4 days. The central dark areas correspond histologically to zones of mineralized cartilage.

(B) In situ hybridization for collagen type X (bright field images, counterstained with hematoxylin/eosin) or osteopontin (dark field images) in metatarsals cultured for 3 or 4 days, as indicated (100×). Similar observations were made in two different experiments.

at day 3, collagen X expression could be seen in only a few cells near the center, and these did not express osteopontin, a marker of more advanced chondrocyte hypertrophy. In contrast, hypertrophic cells in the *PTHR*$^{-/-}$ rudiment at this time already extended abnormally close to the ends of the element. After 4 days, more chondrocytes in the center of the *PTHR*$^{D/-}$ rudiment became hypertrophic and expressed collagen type X and osteopontin. Thus, in comparison with the *PTHR*$^{+/-}$ rudiments, the *PTHR*$^{D/-}$ rudiments demonstrated a striking delay in chondrocyte differentiation in vitro that contrasted sharply with the accelerated differentiation of *PTHR*$^{-/-}$ tissue. Under these ex vivo culture conditions, postmature hypertrophic chondrocytes, expressing osteopontin but no longer collagen X, accumulate in the central portions of the rudiments, which do not undergo vascular invasion or replacement by primary spongiosa. No type I collagen or osteocalcin could be detected by in situ hybridization in these osteopontin-expressing cells (data not shown). These findings suggest that in mediating the action of PTHrP, the PTH/PTHrP receptor exerts both an inhibitory and a stimulatory effect on differentiation of growth plate chondrocytes, depending on the specific signals generated by the receptor.

To further examine the roles of PLC-dependent and -independent signaling pathways on regulation of chondrocyte differentiation by PTH/PTHrP receptors, cultured wild-type metatarsal rudiments were treated for 3 days with either PTH or the direct PKC activator, tetradecanoyl phorbol 13-acetate (TPA). As shown in Figure 5A, the domain of chondrocytes expressing collagen type X and osteopontin was expanded by treatment with TPA (10 nM). In contrast, hypertrophy of chondrocytes was inhibited dramatically by PTH in both wild-type and *PTHR*$^{D/D}$ rudiments, and this inhibitory effect was mimicked by treatment with forskolin, a stimulator of adenylyl cyclase (Figure 5B). These results suggest that PLC/PKC signaling accelerates chondrocyte differentiation. In contrast, PLC-independent signaling (i.e., the cAMP/PKA pathway) slows chondrocyte differentiation, and this pathway likely plays a dominant role in normal regulation of chondrocyte differentiation by PTH/PTHrP receptors.

PLC-Independent Signaling Stimulates Chondrocyte Proliferation

In the mice homozygous for ablation of PTHrP, the rate of chondrocyte proliferation was decreased compared to wild-type, and this decrease in proliferation could be restored to wild-type levels by expression of a constitutively active PTH/PTHrP receptor transgene (Karp et al., 2000). This suggests that the PTH/PTHrP receptor is required for a normal rate of chondrocyte proliferation. To understand which signaling via the PTH/PTHrP receptor regulates chondrocyte proliferation, we determined the percentage of chondrocytes incorporating

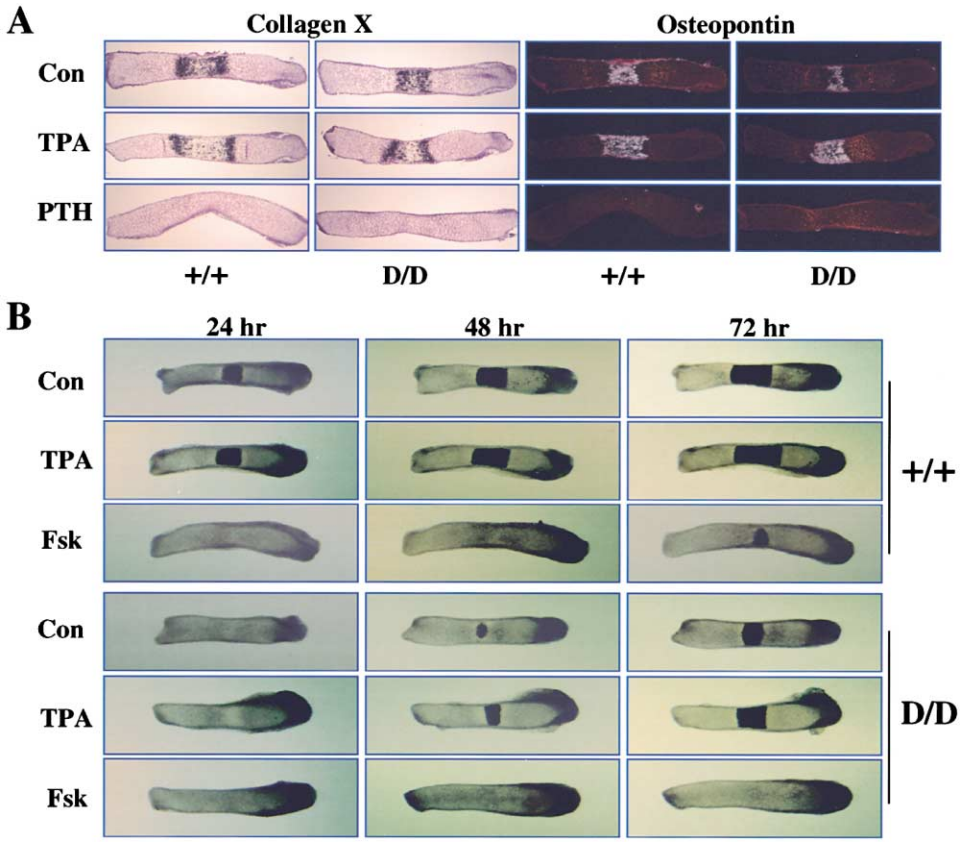

Figure 5. Regulation of Chondrocyte Differentiation in Cultured Metatarsal Rudiments

(A) In situ hybridization of serial sections (100×) for collagen type X (bright field, counterstained with hematoxylin/eosin) or osteopontin (dark field) in E15.5 wild-type or *PTHR*$^{D/D}$ metatarsals cultured for 3 days in medium alone (Con) or in the presence of TPA (10 nM) or rPTH(1–34) (100 nM).

(B) Serial microscopic images (49×) of E15.5 metatarsals from wild-type or *PTHR*$^{D/D}$ mice cultured in medium alone (Con) or in the presence of TPA (10 nM) or forskolin (Fsk, 10 μM) for 24, 48, or 72 hr. Similar observations were made in three independent experiments.

BrdU in the cultured metatarsal rudiments after PTH treatment. In the wild-type rudiments, as expected, PTH(1–34) increased the percentage of proliferating chondrocytes in S phase (Figure 6). A comparable increase in BrdU labeling of chondrocytes was observed in homozygous *PTHR*$^{D/D}$ rudiments (Figure 6). This effect was mimicked by treatment of wild-type rudiments with [Gly^{1}, Arg^{19}]hPTH(1–28), a PTH analog that cannot activate PLC (Takasu et al., 1999b; data not shown). Collectively, these results indicate that PLC-independent (i.e, cAMP/PKA-dependent) signaling via the PTH/PTHrP receptor mediates the proliferative response of growth plate chondrocytes.

Increased Expression of PTH/PTHrP Receptors in DSEL Mutant Chondrocytes

Because Ihh is required for normal levels of chondrocyte proliferation (Karp et al., 2000), we sought effects of the DSEL mutation on hedgehog expression and action. In tibial growth plates of E18.5 *PTHR*$^{D/D}$ mice, however, the patterns and expression levels of mRNAs encoding Ihh and its receptor, patched-1, which is upregulated by Ihh, appeared indistinguishable from those in wild-type littermates (data not shown). To determine whether increased PTH/PTHrP receptor-dependent cAMP/PKA signaling, which appears to be linked to chondrocyte proliferation (Figure 6), might contribute to the increased proliferation observed in chondrocytes of *PTHR*$^{D/D}$ mice (Figure 3), we first examined the level of PTH/PTHrP receptor mRNA by in situ hybridization in tibial growth plates *PTHR*$^{D/D}$ mice. These experiments showed that both the signal intensity and the extent of the domain expressing high levels of PTH/PTHrP receptor mRNA were increased in the growth plates of *PTHR*$^{D/D}$ mice, both at E18.5 and at birth (Figures 7A–7H). A similar increase in intensity and extent of PTH/PTHrP receptor mRNA was observed in the cultured metatarsal rudiments from *PTHR*$^{D/D}$ mice (data not shown). To analyze cell surface expression of the receptor protein in cartilage, we isolated primary chondrocytes from tibia, femur, and fibula at E14.5, a time at which bone formation is very limited in limbs, and measured levels of immunoreactive PTH/PTHrP receptor using anti-receptor antibody. As shown in Figure 7I, the expression of immunoreactive PTH/PTHrP receptors in DSEL mutant chondrocytes was roughly 3-fold that in the wild-type chondrocytes. Northern blot analysis further showed that

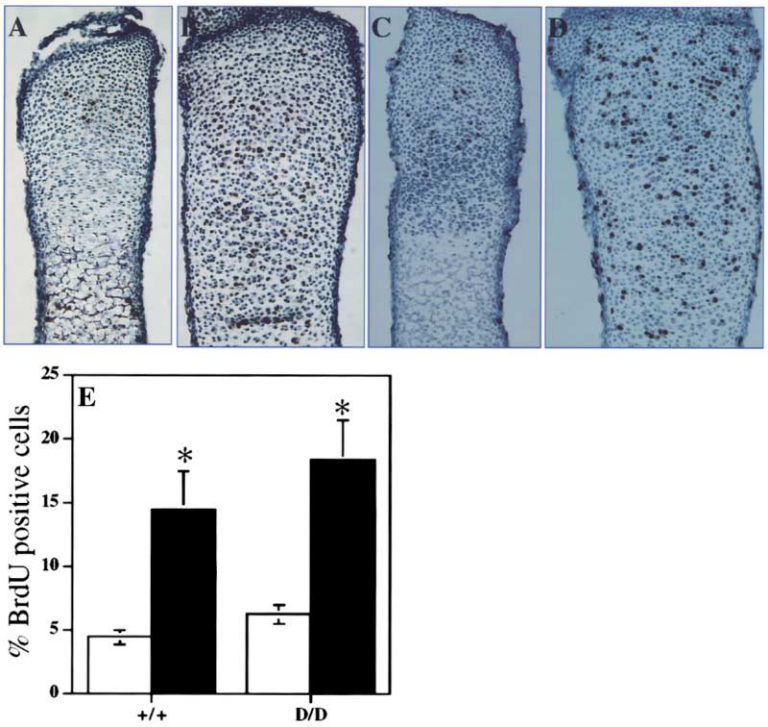

Figure 6. Stimulation of Chondrocyte Proliferation by PTH in Cultured Metatarsal Rudiments

Metatarsals isolated on E15.5 from wild-type (A and B) or *PTHR*$^{D/D}$ (C and D) mice were cultured in the presence of 10 nM rPTH(1–34) (B and D) or vehicle alone (A and C) for 3 days. During the last 6 hr of culture, BrdU (1 mg/ml) was added and incorporated label was detected immunohistochemically. Labeling indices, calculated as the percentages of BrdU-positive nuclei in the zones comprised of periarticular or columnar proliferating chondrocytes, are shown in (E). Bars represent means ± SD for vehicle-treated (open bars) or PTH-treated (filled bars) rudiments. *P $<$ 0.01 (Student's t test), compared to control.

expression of the PTH/PTHrP receptor mRNA in DSEL mutant cartilage also was increased relative to that in the wild-type (data not shown). In parallel cell cultures, the cAMP response to added PTH was much greater in cells from *PTHR*$^{D/D}$ than from wild-type mice (Figure 7J). These data suggest that PLC-dependent signaling normally may suppress the level of PTH/PTHrP receptor expression in growth plate chondrocytes.

Discussion

PTHR$^{D/D}$ mutant embryos display a delay in both the hypertrophy of growth plate chondrocytes and the invasion of capillaries into the hypertrophic cartilage, together with an accumulation of columnar proliferating chondrocytes. These changes are observed early in the development of a wide range of bones formed via the endochondral sequence. The expansion of proliferating columnar chondrocytes results from both increased proliferation and delayed differentiation into hypertrophic chondrocytes. It is notable also that the growth plates of *PTHR*$^{D/D}$ mice do not exhibit an accumulation of hypertrophic chondrocytes or mineralized cartilage, nor is there an apparent abnormality in formation of primary spongiosa. Thus, we can conclude also that PLC activation via the PTH/PTHrP receptor is not required for the resorption of cartilage matrix, local vascular invasion, and subsequent formation of primary spongiosa involved in this orderly developmental progression.

These findings in *PTHR*$^{D/D}$ mutant mice provide insight into the physiologic regulation of chondrocyte proliferation and differentiation by PLC-dependent versus PLC-independent PTH/PTHrP receptor signaling. In mediating the actions of PTHrP, chondrocytic PTH/PTHrP receptors act to slow chondrocyte differentiation and to extend or accelerate chondrocyte proliferation. This has been inferred from previous analyses of the phenotypes of mice in which genes encoding PTHrP or PTH/PTHrP receptors were ablated (Karaplis et al., 1994; Lanske et al., 1996), in which PTHrP was overexpressed in chondrocytes (Weir et al., 1996), or in which constitutively active PTH/PTHrP receptors could be shown to rescue the growth plate abnormalities in PTHrP null animals (Schipani et al., 1997). The accelerated proliferation and delayed differentiation are augmented in *PTHR*$^{D/D}$ mice, which provides strong evidence that they are mediated via PLC-independent signaling mechanisms.

The effect of the DSEL mutation on the size of the hypertrophic chondrocyte layer changes over time. Early in development, a smaller hypertrophic zone in *PTHR*$^{D/D}$ mice is found (e.g., Figure 2A, metatarsals at E14.5 and E15.5). This observation presumably reflects the delay in exit of chondrocytes from the proliferative pool. After hypertrophic cells start to die in normal mice (e.g., the tibiae at E14.5 in Figure 2B), there are actually more hypertrophic chondrocytes in the *PTHR*$^{D/D}$ mice, presumably because the disappearance of hypertrophic chondrocytes has not yet started in these mice. In contrast, in E15.5 and E18.5 mice, the size of the hypertrophic zones in wild-type and *PTHR*$^{D/D}$ mice are of roughly similar size. At any time, the extent of the hypertrophic zone depends upon the rate at which new hypertrophic cells are formed minus the rate at which hypertrophic cells die or otherwise lose their character as hypertrophic cells. (The ultimate fate of hypertrophic chondrocytes still has not been directly demonstrated, though apoptosis is certainly an important component.) Thus, the normal size of the hypertrophic pool at later times presumably reflects a balance of hypertrophic cell production and loss.

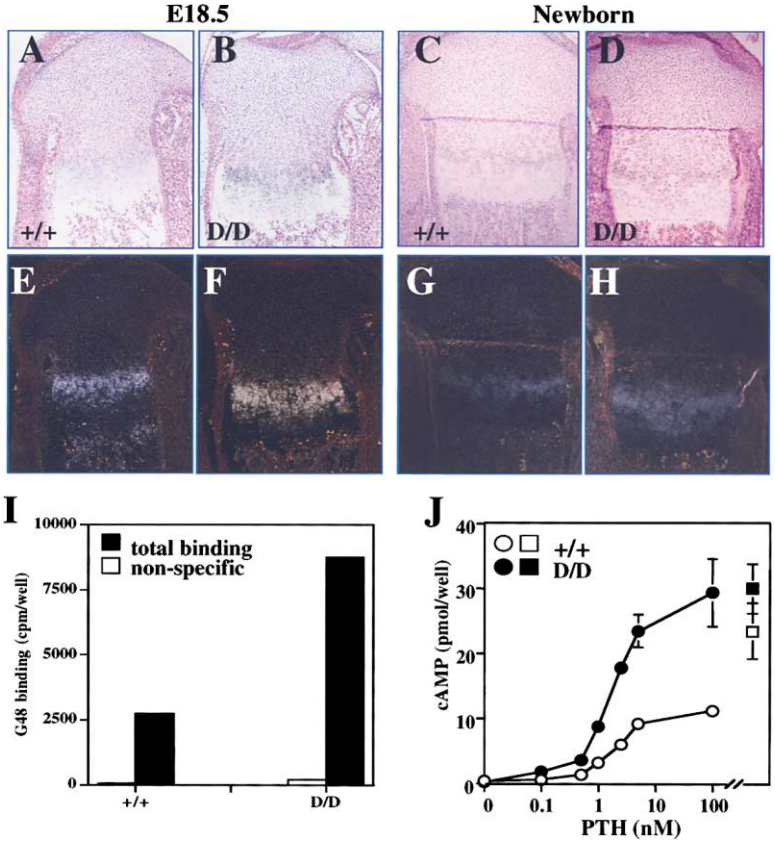

Figure 7. PTH/PTHrP Receptor Expression in Tibial Growth Plates

(A–H) In situ hybridization for PTH/PTHrP receptors was performed on longitudinal sections through wild-type and *PTHR*$^{D/D}$ proximal tibias at two stages of development (as indicated). After hybridization, sections were stained with hematoxylin and eosin. (A–D) bright field; (E–H) dark field. PTH/PTHrP receptor is expressed in a much broader domain and signal is more intense, both at E18.5 (B and F) and in newborn (D and H) tibias, in *PTHR*$^{D/D}$ mutants. Similar observations were made in two independent litters from each stage of development examined.

(I) Assessment of cell surface PTH/PTHrP receptor protein in primary chondrocytes. Primary chondrocytes isolated from wild-type or *PTHR*$^{D/D}$ mutant femur, tibia, and fibula at E14.5 were plated in 24-well plates. After confluence, G48 anti-receptor antibody binding analysis was performed. Results are expressed as means ± SEM of triplicates (cpm bound/well). Total (filled bars) and nonspecific (open bars) binding was 2796 ± 76 and 60 ± 12 for the wild-type and 8755 ± 192 and 229 ± 41 for the *PTHR*$^{D/D}$ mutants, respectively. Protein content (μg/well) was 43.4 ± 3.2 and 48.5 ± 1.5 for the wild-type and *PTHR*$^{D/D}$ mutants, respectively. Similar results were obtained in three independent experiments.

(J) Cyclic AMP stimulation by PTH in primary chondrocytes. Primary chondrocytes were isolated as above, and cultured in 24-well plates for 1 week and then exposed to rPTH(1–34) at the indicated concentrations for 10 min in the presence of 3-isobutyl-1-methylxanthine (0.5 mM). The responses of wild-type (open symbols) and *PTHR*$^{D/D}$ (filled symbols) cells to forskolin (10 μM, squares) were also assessed. Results are expressed as means ± SEM of triplicates for pmol/well of cAMP. Similar results were obtained in two experiments.

The action of TPA to accelerate chondrocyte differentiation in metatarsal bone rudiments from both wild-type and *PTHR*$^{D/D}$ mice suggests that the actions of the DSEL PTH/PTHrP receptor does, in fact, reflect a decrease in PLC signaling. Our results in vivo, as well as the inhibitory actions of forskolin and of the cAMP-selective analog [Gly^{1},Arg^{19}]hPTH(1–28) upon differentiation observed in metatarsal rudiments, are most consistent with primary involvement of the cAMP/PKA pathway in these actions, although it is possible that other PTH/PTHrP-dependent signaling events occur and can be transmitted via DSEL receptors. A major role for cAMP/PKA signaling is highlighted also by the recent observation, in a chimeric mouse model, that growth plate chondrocytes lacking $G_s\alpha$ exhibit accelerated differentiation (Chung et al., 2000).

In contrast, PLC-dependent signaling via the PTH/PTHrP receptor appears to restrain proliferation of hypertrophic chondrocytes and to stimulate their progression toward hypertrophic differentiation. These actions are opposite to the dominant net responses to PTH/PTHrP activation, as revealed by receptor ablation or constitutive activation, which suggests that PLC activation plays a modulating role that counteracts the major effects of the cAMP/PKA pathway. Our results suggest that this effect of PLC activation may be mediated, in part, by decreased expression of the PTH/PTHrP receptor and a corresponding reduction in cAMP/PKA activation. On the other hand, our observation of a direct action of the PKC activator TPA, to augment differentiation in metatarsal rudiments in vitro, leaves open the possibility that these modulating effects may result from other downstream transcriptional responses to PLC/PKC activation in chondrocytes as well. The opposition between adenylyl cyclase and PLC activation via the same receptor appears paradoxical but may serve to provide increased opportunities for regulation. For example, since PLC activation via PTH/PTHrP receptors, relative to that of adenylyl cyclase, is more efficient at higher concentrations of ligand (Guo et al., 1995), we might speculate that PLC activation plays a greater role near the articular end of the growth plate, where endogenous PTHrP concentrations are highest, and could serve to "smoothen" the functional gradient of PTHrP receptor-mediated developmental regulation.

Thus, activation of multiple G proteins by the PTH/PTHrP receptor in vivo has important physiologic consequences. By extension, it seems likely that other G protein-coupled receptors also may use multiple signaling pathways to regulate important physiologic events in vivo.

Experimental Procedures

Cells

J1 embryonic stem (ES) cells from 129/SvJ mice were maintained on a layer of irradiated mouse embryonic fibroblasts in ES cell medium, as described previously (Li et al., 1992).

Construction of Targeting Vector

To construct the targeting vector, the 13.5 kb EcoRI/EcoRI fragment of the murine PTH/PTHrP receptor gene was excised from a single phage and subcloned into pUC19 (BioLabs) that already contained a PGKtk gene (2.7 kb HindIII/EcoRI fragment from pPNT) inserted at its KpnI site (Lanske et al., 1996). A 4.5 kb KpnI/XhoI fragment from the 13.5 kb EcoRI/EcoRI subclone then was used to replace the EKKY sequence in the second intracellular loop of the PTH/PTHrP receptor with the DSEL mutation (*, Figure 1A). PGKneo resistance expression cassette (1.8 kb XhoI/XbaI fragment from pPNT) was cloned into the pGEM7 vector with flanking 34 bp loxP sequences, and then inserted at an EcoRV site. The completed targeting vector (Figure 1) consisted of a 13.5 kb genomic sequence of the PTH/PTHrP receptor gene incorporating the desired DSEL mutation, a nearby intronic PGKneo gene (flanked by loxP sites to enable positive selection in G418), and a 5′ PGKtk gene to allow for negative selection with gancyclovir.

Gene Targeting and Blastocyst Injections

The targeting vector (45 μg) was linearized with SalI and then electroporated into 10^7 J1 ES cells (Bio-Rad Gene Pulser, 0.24 kV, 0.5 mF). Restriction analysis of DNA from 168 clones resistant to both G418 and gancyclovir revealed that nine clones had undergone the desired recombination event. From three of these properly targeted clones, the PGKneo resistance cassette was removed by Cre-mediated recombination, accomplished by electroporating 10^6 cells with 30 μg of an uncut CMV-Cre recombinase expression vector (kindly provided by Dr. O'Gorman, The Salk Institute for Biological Studies). Ten percent of the Cre-transfected ES clones were correctly targeted (59 of 560 clones screened). Four of these correctly targeted ES cell lines were microinjected into C57BL/6 blastocysts, which were implanted into pseudopregnant CD-1 female recipients. Male chimeras were then mated with C57BL/6 females. Germline transmission was identified by Southern blot hybridization of tail DNA. Animals were maintained in facilities operated by the Center for Comparative Research of the Massachusetts General Hospital, and all studies performed were approved by the institution's Subcommittee on Research Animal Care.

Skeletal Staining

Alizarin red and Alcian blue staining was performed as previously described (Komori et al., 1997). Embryos at E18.5 were fixed at 4°C for 48 hr in ethanol and then for 24 hr in acetone. Staining was performed for 4 days at room temperature. After washing with 95% ethanol, the skeleton was cleared in 1% KOH and taken through graded steps into 100% glycerol.

Histologic Analysis and In Situ Hybridization

Embryos at stages E12.5–E18.5, as well as tibiae from postnatal day 1 through day 120, were dissected in ice-cold PBS, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Horizontal coronal sections (5 μm) were cut and stained with hematoxylin and eosin. In situ hybridization was performed as described previously (Chung et al., 2001), using complementary ^{35}S-labeled riboprobes for mouse type X collagen, mouse osteopontin, and rat PTH/PTHrP receptor (Calvi et al., 2001).

Analysis of BrdU Incorporation

Pregnant mice were injected intraperitoneally with 100 μg bromodeoxyuridine (BrdU) and 12 μg fluorodeoxyuridine (FdU) per gram of body weight between 2 and 48 hr before sacrifice. Embryos were fixed and embedded in paraffin. Sections (5 μm) containing tibial growth plates were used for immunodetection of BrdU, according to the manufacturer's protocols (Zymed Laboratories). To determine the rate of cell proliferation, photomicrographs were taken at 200× magnification, and labeled and unlabeled chondrocytes were counted in the periarticular proliferating, columnar proliferating, and hypertrophic zones of the growth plate. Cells in three adjacent sections from three independent wild-type (+/+) and homozygous DSEL mutant (*PTHR$^{D/D}$*) mice were scored. Differences in percentages of labeled nuclei were assessed using Student's t test.

Primary Cell and Explant Culture

Primary proximal tubule cells were isolated as described previously (Janulis et al., 1993). Briefly, kidneys from *PTHR$^{+/+}$*, *PTHR$^{+/D}$*, and *PTHR$^{D/D}$* mice were bisected, the capsule and medulla were removed, and the cortex then was minced with sharp scissors and subjected to digestion for 1 hr with type 1 collagenase (Worthington Biochemical Corporation; 1 mg/ml) in DMEM medium. Cell aggregates then were dispersed by repeated pipetting, and the resulting cell suspension was passed through an ice-cold mesh (75 μm) before centrifugation at 500 g for 5 min. After washing the digested cells twice in DMEM medium, they were applied to a preformed Percoll gradient and centrifuged at 1000 rpm for 1 hr at 4°C to obtain purified primary proximal tubule cells.

Chondrocytes from embryonic limbs were isolated as described previously (Guo et al., 2001). Femur, tibia, and fibula at E14.5 were dissected. After mincing with sharp scissors, the tissue was digested for 8 hr with a mixture of type 1 and type 2 collagenase (1:2) in α-MEM medium at a total concentration of 1 mg/ml. Primary chondrocytes were plated in 6-well or 24-well plates and maintained in α-MEM medium supplemented with 8% fetal bovine serum and antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin).

Embryonic metatarsals were dissected and cultured as described previously (Haaijman et al., 1999). The second, third, and fourth metatarsals from each embryonic (E15.5) hindlimb were dissected under sterile conditions. Each metatarsal was cultured in 300 μl of α-MEM medium in a separate well of a 24-well plate for up to 4 days without changing the medium. Fresh agonists were added daily in small volumes. During culture, mineralized cartilage could be recognized under an inverted microscope as a dark area developing in the center of the hypertrophic zone, flanked at each end by a proliferative zone extending to the end of the rudiment.

Cyclic AMP and IP Assay

Primary proximal tubule cells obtained from *PTHR$^{+/+}$*, *PTHR$^{+/D}$*, or *PTHR$^{D/D}$* mice were plated in 24-well or 6-well dishes and cultured in DMEM medium (prepared in the Massachusetts General Hospital media kitchen). When confluent, cells were treated with rPTH(1–34) amide for 20 or 40 min for determination of cAMP (Guo et al., 1997), or inositol triphosphate (IP3) (RIA kit, TRK1000; Amersham Life Science).

Cell Surface Expression of the PTH/PTHrP Receptor

A PTH/PTHrP receptor-specific antibody was used as previously described (Iida-Klein et al., 1997) to measure cell surface expression of wild-type and DSEL mutant receptors. Briefly, primary proximal tubule cells and chondrocytes obtained from wild-type or DSEL mutant mice were cultured in 24-well plates. When confluent, cells were washed twice with PBS, incubated at room temperature for 2 hr with sheep anti-rat PTH/PTHrP receptor-specific antibody G48 (1:500 in PBS with 5% FBS), washed, and then incubated successively with second antibody (rabbit anti-sheep IgG; Kirkegaard and Perry Laboratories) and third antibody (goat anti-rabbit ^{125}I-IgG; DuPont NEN). Washed cells then were solubilized in 0.5 ml of 0.2 N NaOH/0.1% SDS for determination of radioactivity.

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